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Short Article

Interactions between Inner Membrane Proteins in Donor and Recipient Cells Limit Conjugal DNA Transfer

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Summary

Conjugation enables horizontal transmission of DNA among bacteria, thereby facilitating the rapid spread of genes such as those conferring resistance to antibiotics. Cell-cell contact is required for conjugative DNA transfer but does not ensure its success. The presence of certain plasmids in potential recipient cells inhibits redundant transfer of these plasmids from competent donors despite contact between donor and recipient cells. Here, we used two closely related integrating conjugative elements (ICEs), SXT and R391, to identify genes that inhibit redundant conjugative transfer. Cells containing SXT exclude transfer of a second copy of SXT but not R391 and vice versa. The specific exclusion of SXT and R391 is dependent upon variants of TraG and Eex, ICE-encoded inner membrane proteins in donor and recipient cells, respectively. We identified short sequences within each variant that determine the exquisite specificity of self-recognition; these data suggest that direct interactions between TraG and Eex mediate exclusion.

Introduction

Integrative and conjugative elements (ICEs) are an emerging and diverse class of prokaryotic mobile genetic elements. These elements are transmissible via conjugation and integrate into their hosts' chromosomes. In addition to containing DNA sequences coding for self-transmission and maintenance, ICEs impart a wide range of properties to their hosts and are important vehicles for the dissemination of a variety of antibiotic resistance genes in both gram-negative and gram-positive bacteria (Burrus and Waldor, 2004b).

SXT (100 kb) and R391 (89 kb) are related ICEs that were originally detected in clinical isolates of *Vibrio cholerae* and *Providencia rettgeri*, respectively (Coetzee et al., 1972; Waldor et al., 1996). SXT confers resistance to the antibiotics sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin, while R391 carries resistances to kanamycin and mercury. Comparison of the complete nucleotide sequences of R391 and SXT revealed that they share 95% identity over 65 kb of "backbone" genes whose products mediate the integration/excision, regulation, and conjugative transfer of the respective elements (Beaber et al., 2002a). Excision of SXT and R391 from the chromosome yields circular but nonreplicating forms of the elements that are postulated to be templates for production of the single-stranded DNA that is transferred to recipient cells via conjugation (Hochhut and Waldor, 1999).

The SXT and R391 conjugal transfer genes are related to those found in the F plasmid. Studies of the F plasmid have yielded key insights into the biology of bacterial conjugation. Conjugation requires cell-to-cell contact where the outer membranes of donor and recipient cells become closely apposed at conjugative junctions (Samuels et al., 2000). Cell-cell contacts are initiated by pili, cell-surface appendages present on donor cells encoded by the conjugative plasmid. Initial cell-cell contacts are subsequently stabilized through a poorly understood process that generates cell aggregates that are resistant to shear forces (Manning et al., 1981). The F-encoded inner membrane protein TraG is required for mating-pair stabilization (Firth and Skurray, 1992; Lawley et al., 2003). Concomitant with mating-pair stabilization, one strand of the F plasmid is cleaved at its origin of transfer. Subsequently, transport of single-stranded plasmid DNA through the mating pore to the recipient cell ensues.

Interactions between donor and recipient cells does not always lead to DNA transfer, as recipient cell exclusion proteins can interfere with this process. Two types of exclusion proteins have been identified, although their mechanisms of action remain poorly understood. Surface exclusion is mediated by outer membrane proteins; TraT, encoded by the F plasmid, is thought to impede the conjugative pilus from contacting the recipient cell (Achtman et al., 1977). Entry exclusion is mediated by proteins that localize to the inner membrane. The entry exclusion proteins TraS from F and Exc from R144 have been shown to abort conjugation after stable mating pairs have been formed, presumably by inhibiting DNA transfer from the donor (Hartskeerl and Hoekstra, 1984; Ou, 1975).

To date, exclusion has been described for conjugative plasmids. Here we describe an exclusion system carried by ICEs. We found that cells containing SXT exclude transfer of a second copy of SXT but not R391 and vice versa. EexS and EexR, inner membrane proteins encoded by SXT and R391, respectively, are sufficient for specifying exclusion activity in recipients. We identified the inner membrane mating pair formation protein TraG as the donor cell component required for Eex exclusion. We present genetic evidence that supports a model in which direct interactions between TraG and Eex variants mediate exclusion.

Results

eexS and *eexR* Provide SXT- and R391-Specific Exclusion Activity, Respectively

Although R391 and SXT have nearly identical conjugation genes, they do not exclude each others' transfer. The frequency of transfer of R391 to a cell harboring SXT is the same as the frequency of its transfer to a cell lacking SXT (Hochhut et al., 2001). We first examined whether the presence of SXT in a cell diminishes its

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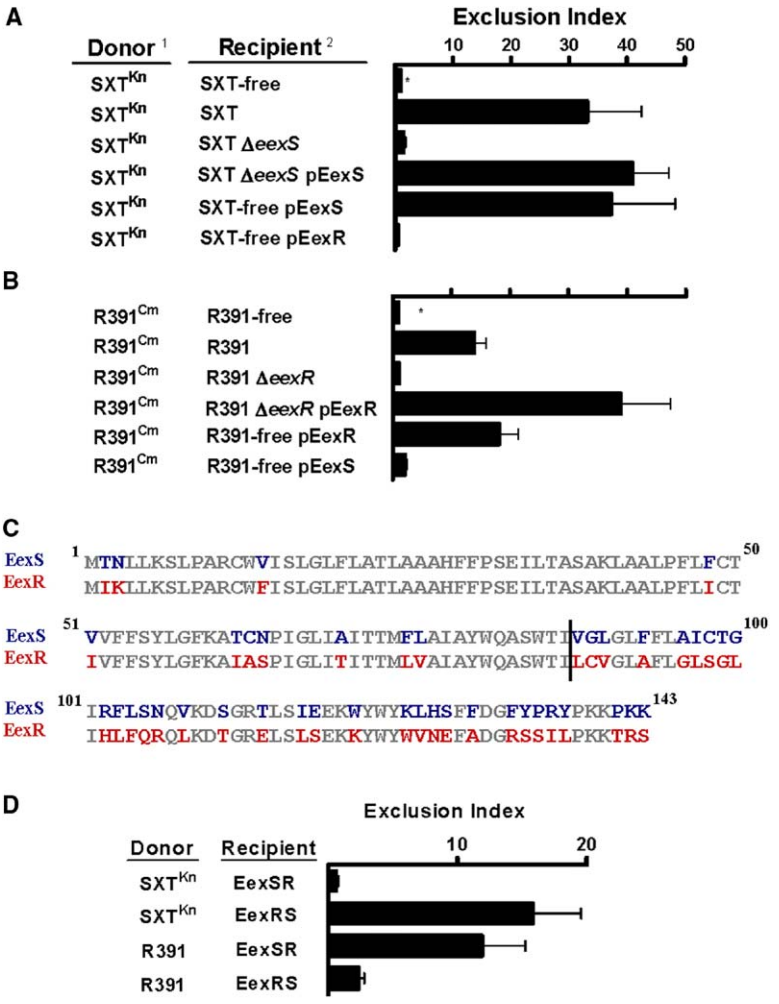

Figure 1. *eexS* and *eexR* Provide SXT- and R391-Specific Exclusion Activity

The exclusion activity of recipient cells for SXT or R391 transfer is expressed as an exclusion index, which is the ratio of an element's transfer frequency to an ICE-free recipient divided by the transfer frequency to the indicated recipient. Each bar represents the mean of three experiments, with error bars indicating the standard deviation.

(A) Exclusion index for SXT^{Kn} transfer (EI_S). [1]All donor strains are derivatives of *E. coli* MG1655. [2]All recipient strains are derivatives of CAG18439 (MG1655 *lacI*3042::Tn10 (Singer et al., 1989). *EI_S and EI_R of element-free recipients are defined as one.

(B) Exclusion index for $R391^{Cm}$ transfer (EI_R).

(C) Alignment of the predicted amino acid sequences of EexS and EexR. Blue and red highlight SXT- and R391-specific amino acids. The vertical black line indicates the point of the junction between EexS and EexR amino acid sequences in the chimeric proteins EexSR and EexRS.

(D) The chimera EexRS contains the amino terminus of EexR and the carboxyl terminus from EexS while EexSR contains the amino terminus of EexS and the carboxyl terminus of EexR. Donors harboring either R391 or SXT^{Kn} were mated with ICE-free recipients expressing either EexSR or EexRS from a plasmid.

capacity to acquire a second copy of the element. To test this, we constructed a differentially marked SXT in which the chloramphenicol resistance gene was replaced with a kanamycin resistance gene (SXT^{Kn}). The frequency of transfer of SXT^{Kn} to an SXT-free *E. coli* strain was 34 times greater than to an isogenic recipient strain harboring SXT. This is shown in Figure 1A as the exclusion index for SXT (EI_S); EI_S was defined as the frequency of transfer of SXT^{Kn} to an SXT-free recipient divided by the frequency of transfer of SXT^{Kn} to a recipient of interest; consequently, a high exclusion index indicates poor ICE transfer. Similarly, transfer of a differentially marked R391 ($R391^{Cm}$) to a recipient containing R391 was lower than to a recipient lacking R391 (EI_R = 14, Figure 1B).

We mapped the SXT gene responsible for providing exclusion activity in the recipient cell using a set of *E. coli* strains harboring a series of SXT deletion mutants (Beaber et al., 2002b). The frequency of transfer of SXT^{Kn} to a recipient harboring SXT Δ*s079* was the same as that to a recipient that lacked SXT (Figure 1A), indicating that *s079*, a hypothetical gene (Beaber et al., 2002b), was required for SXT exclusion. *s079* appears to function only in recipient cells and is dispensable for ICE transfer from donor cells since transfer frequencies of SXT and SXT Δ*s079* were the same (data not shown). Based on evidence presented below suggesting that *s079* encodes an entry exclusion factor, this gene was renamed *eexS* (entry exclusion for SXT). The exclusion defect of the SXTΔ*eexS* recipient was complemented by expression of *eexS* from a plasmid. Furthermore, *eexS* was sufficient for SXT exclusion, as a recipient lacking SXT but expressing *eexS* from a plasmid had an EI_S of 37 (Figure 1A).

R391 contains a hypothetical gene, *cds89*, that has significant similarity to *eexS*. *eexS* and *cds89* are located in the same relative position within the SXT and R391 genomes. We deleted *cds89* from R391 to test whether this gene accounts for R391 exclusion. The R391 exclusion index (EI_R) for a recipient harboring R391 Δ*cds89* was 1.5, similar to a recipient lacking R391, indicating that *cds89* (here named *eexR*) plays a critical role in R391 exclusion (Figure 1B). The exclusion deficiency of the R391 Δ*eexR* recipient was complemented by expression of *eexR* from a plasmid. Moreover, expression of *eexR* from a plasmid in an R391-free recipient was sufficient to generate R391 exclusion (Figure 1B). Thus, *eexS* and *eexR* are the necessary and suffi-

cient ICE components that mediate SXT and R391 exclusion, respectively. Furthermore, the exclusion activities of *eexS* and *eexR* are element specific. A recipient expressing *eexS* from a plasmid did not exclude R391, and a recipient expressing *eexR* from a plasmid did not exclude SXT (Figure 1).

EexS and EexR Exclusion Specificity Is Determined by Their Carboxyl Regions

The EexS and EexR amino acid sequences are 77% similar and most of the sequence differences lie in their carboxy-terminal regions (Figure 1C). Eex protein chimeras were constructed by exchanging the 3′ halves of the two *eex* genes (see Figure 1C) to test the possibility that the carboxyl regions of the Eex proteins determine their exclusion specificities. A recipient expressing the EexRS chimera, composed of the amino terminus of EexR and the carboxyl terminus of EexS, excluded SXT but not R391. Conversely, a recipient expressing EexSR, a protein composed of the amino terminus of EexS and the carboxyl terminus of EexR, excluded R391 but not SXT (Figure 1D). These findings indicate that the Eex carboxyl region amino acid sequence dictates exclusion specificity.

TraG Is the Exclusion Target in Donor Cells

After determining that *eex* variants mediate entry exclusion in recipient cells, we investigated which component in donor cells mediates ICE exclusion. We first tested whether the presence of *eexS* in SXT in the donor is required for exclusion. Deletion or overexpression of *eexS* in the donor cell had no influence on SXT exclusion from recipients expressing *eexS* (data not shown). Thus, exclusion is not mediated by homotypic interactions between Eex proteins present in both donor and recipient cells; instead, some other SXT gene product(s) must act in the donor cell to mediate exclusion.

A genetic screen that took advantage of *eex* specificity was used to identify the ICE gene product that acts in donor cells to mediate exclusion. Strains containing various SXT deletion mutants (Beaber et al., 2002b), along with intact R391, were used as donors and mated with strains expressing either *eexS* or *eexR*. We assessed whether SXT transfer from these donor strains was inhibited by *eexR* rather than *eexS*, as would occur if a protein conferring exclusion specificity was supplied by R391 rather than SXT. This screen suggested that *traG*, an ortholog of the F-plasmid mating pair formation gene, contributes to *eex*-mediated exclusion. Transfer of SXT Δ*traG* from a donor containing R391 was inhibited when recipient cells expressed *eexR* and not when they expressed *eexS.* Similarly, R391 Δ*traG* transfer from a donor also containing SXT was excluded by a recipient expressing *eexS* and not by a recipient expressing *eexR* (data not shown).

Complementation experiments confirmed that EexS- and EexR-mediated exclusion was dependent upon the TraG allele employed in the donor for ICE transfer. SXT Δ*traG* and R391 Δ*traG* are nontransmissible (Beaber et al., 2002b), but provision of $traG_S$ or $traG_R$ from a plasmid restored transmission of these elements to wild-type levels (data not shown). Transfer of SXT Δ*traG* and R391 Δ*traG* from strains containing $pTraG_S$ was excluded by a recipient expressing *eexS* but not by a recipient expressing *eexR* (Figure 2A). Similarly, transfer of SXT Δ*traG* and R391 Δ*traG* from strains containing $pTraG_R$ was excluded by a recipient expressing *eexR* but not by a recipient expressing *eexS* (Figure 2B). These findings suggest that EexR and EexS in recipient cells can recognize and discriminate between the TraG proteins present in donor cells. Overexpression of $traG_S$ in a recipient cell did not alter its capacity to exclude SXT, confirming that the role of TraG in exclusion is only relevant when it is produced in the donor cell (data not shown).

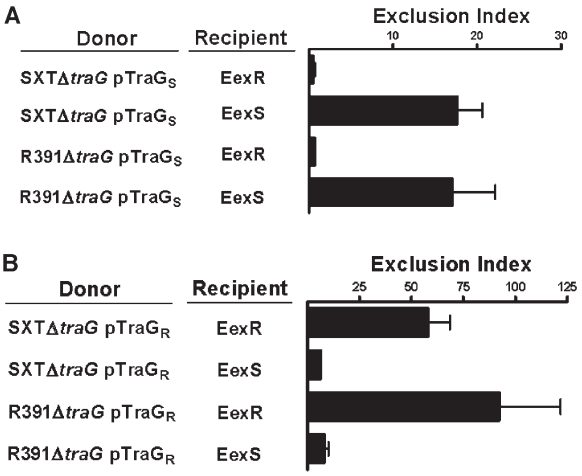

Figure 2. TraG Is the Exclusion Target in Donor Cells

Donor strains containing SXTΔ*traG* or R391Δ*traG* were complemented with a plasmid expressing TraG derived from SXT (A) or R391 (B). Each donor strain was mated with an ICE-free recipient expressing *eexS* or *eexR* from a plasmid. Entry exclusion indices represent the mean and standard deviations derived from three experiments.

Three Amino Acids in TraG Determine Exclusion Specificity

Alignment of the predicted amino acid sequences of $TraG_S$ and $TraG_R$ revealed that these two 1189 amino acid proteins are 98% identical. Eight of the 18 amino acid differences between $TraG_S$ and $TraG_R$ are clustered between amino acids 606 and 650 (Figure 3A), and we hypothesized that these variations account for the observed exclusion specificity of these proteins. To test this idea, we engineered chimeric SXT and R391 in which we exchanged the sequences from the variant regions (amino acids 606–650) of $TraG_S$ and $TraG_R$, yielding SXTtraG-1 and R391traG-2 (Figure 3A). This sequence exchange reversed the exclusion specificity of the chimeric ICEs; SXTtraG-1 was excluded by a recipient expressing *eexR*, and R391traG-2 was excluded by a recipient expressing *eexS* (Figure 3B). Construction of additional chimeric TraG proteins revealed that merely changing three amino acids in TraG was sufficient to alter exclusion specificity. The $TraG_R$ amino acid residues at positions 606–608 (Thr-Asp-Asp) were changed to the $TraG_S$ amino acid residues found in these positions (Pro-Gly-Glu), creating the chimera

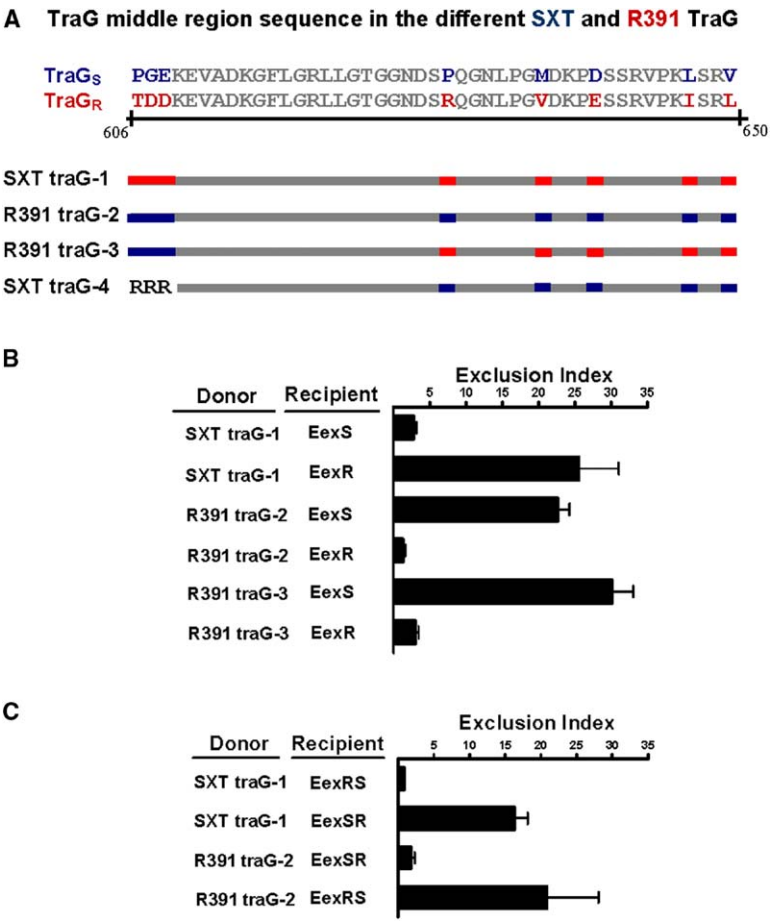

Figure 3. Three Amino Acids in TraG Are Necessary for Exclusion Activity and Specificity

(A) Alignment of amino acids 606–650 of $TraG_S$ and $TraG_R$. SXT-specific amino acids are shown in blue and R391-specific amino acids are shown in red. SXT and R391 chimeric TraG constructs are depicted.

(B and C) Exclusion indices for the transfer of SXT- or R391-containing chimeric TraG into recipients expressing the indicated Eex protein. Entry exclusion indices represent the mean and standard deviation from three experiments.

R391traG-3 (Figure 3A); transfer of R391traG-3 was excluded by a recipient expressing *eexS* and not by a recipient expressing *eexR* (Figure 3B).

We tested whether SXTtraG-1 and R391traG-2 would be excluded by the chimeric Eex proteins discussed above, to confirm that the TraG-1 and TraG-2 proteins acted similarly to $TraG_R$ and $TraG_S$, respectively. Indeed, SXTtraG-1 was excluded from a recipient expressing the chimeric exclusion protein EexSR, and R391traG-1 was excluded from a recipient expressing the EexRS chimera (Figure 3C). These findings demonstrate the allelic specificity of the interactions between TraG and Eex variants.

The Exclusion and Mating-Pair Formation Activities of TraG Are Separable

The observation that particular TraG amino acids are important for entry exclusion allowed us to examine whether the entry exclusion and mating pair formation activities of this protein are separable. We changed the three $TraG_S$ residues important for exclusion activity, positions 606–608, to three arginine residues to generate SXTtraG-4 (Figure 3A). SXT and SXTtraG-4 had virtually identical frequencies of transfer to a recipient lacking SXT (5×10^{-5} versus 4×10^{-5}, respectively), indicating that these changes to the $TraG_S$ sequence did not alter its ability to promote mating pair formation. In contrast, these alterations in the $TraG_S$ sequence abolished the protein's exclusion activity. SXTtraG-4 was not excluded by recipients expressing either *eexS* ($EI_S = 1$) or *eexR* ($EI_S = 1$). These findings demonstrate that TraG exclusion and mating pair formation activities are separable.

EexS and $TraG_S$ Localize to the Inner Membrane

Bioinformatic analyses of the predicted EexS and EexR amino acid sequences using several algorithms, including PSORT (Nakai and Kanehisa, 1991) and HMMTOP (Tusnady and Simon, 2001), suggested that these proteins localize to the bacterial inner membrane. Cell fractionation experiments confirmed this prediction. Nearly all of EexS-His_6, a functional C-terminal His_6-tagged EexS, was found in the inner membrane cell fraction (Figure 4). The characterized plasmid-encoded entry exclusion proteins also localize to the inner membrane (Haase et al., 1996; Hartskeerl et al., 1985a; Jalajakumari et al., 1987; Pohlman et al., 1994).

Orthologs of $TraG_S$ and $TraG_R$, such as the F-plasmid TraG, have been shown to localize to the inner membrane (Firth and Skurray, 1992). $TraG_S$ was also found to localize to the inner membrane; nearly all of $TraG_S$-His_6, a functional C-terminal His_6-tagged $TraG_S$, was found in the inner membrane cell fraction (Figure 4). Thus, our findings suggest that SXT and R391 exclu-

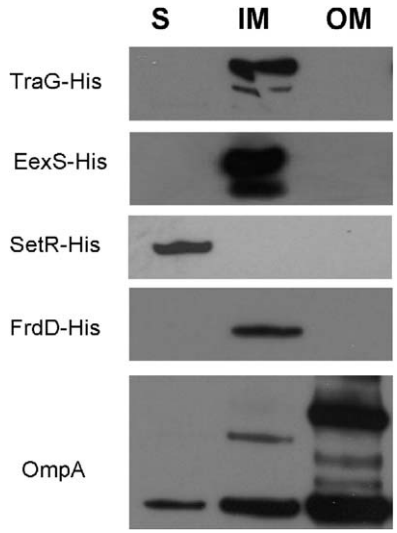

Figure 4. EexS and $TraG_S$ Localize to the *E. coli* Inner Membrane

Localization of EexS-His_6 and $TraG_S$-His_6 in *E. coli* cell fractions: S, soluble; IM, inner membrane; OM, outer membrane. SetR-His_6 (Beaber and Waldor, 2004), a known cytoplasmic protein; FrdD-His (Westenberg et al., 1993), a known inner membrane protein; and OmpA (Freudl et al., 1985), a known outer membrane protein were used as controls to verify the fractionation. TraG-His_6 (128 kDa), EexS-His_6 (18 kDa), SetR-His_6 (30 kDa), and FrdD-His_6 (34 kDa) proteins were detected in each fraction using an anti-His antibody, and OmpA (35 kDa and 62 kDa) was detected using rabbit anti-OmpA serum (Prasadarao et al., 1996). Two protein species were detected for the epitope-tagged EexS expressed from pEexS, a derivative of pBAD. Additional studies revealed that these two protein species resulted from two alternative translation start sites in the EexS plasmid construct and not from protein processing. The larger product (~18 kDa) is derived from the Shine-Dalgarno sequence from the pBAD vector, while the smaller product (~16 kDa) arose from a Shine-Dalgarno sequence within the cloned EexS sequence.

sion is mediated by interactions between inner membrane proteins present in donor (TraG) and recipient (Eex) cells.

Discussion

Like cells harboring certain conjugative plasmids, cells containing integrated SXT or R391 inhibit redundant transmission of these ICEs from donor cells. ICE exclusion is specific; despite the overall sequence similarity of SXT and R391, cells containing SXT exclude transfer of a second copy of SXT but not R391 and vice versa. The specific exclusion of SXT and R391 from cells harboring these ICEs was dependent upon variants of ICE *traG* and *eex* genes, which encode inner membrane proteins, in donor and recipient cells, respectively. Our data are consistent with a model in which direct interactions between cognate TraG and Eex proteins dictate exclusion specificity. Thus, we propose that a form of cell-cell recognition mediated by interactions between inner membrane proteins regulates conjugal gene transfer.

Even though *eexS* and *eexR* lack homologs in the database, these genes are functionally similar to plasmid-encoded entry exclusion genes. These plasmid genes code for inner membrane proteins that function in recipient cells to prevent redundant transfer but are dispensable for plasmid transfer from donor cells (Achtman et al., 1980; Furuya and Komano, 1994; Haase et al., 1996; Pohlman et al., 1994). As with *trbK* from plasmid RP4 and *eex* from pKM101 (Haase et al., 1996; Pohlman et al., 1994), expression of *eexS* or *eexR* in recipient cells is both necessary and sufficient to exclude SXT or R391 from the cell. The reported entry exclusion proteins (Finlay and Paranchych, 1986; Furuya and Komano, 1994; Haase et al., 1996) are highly diverse in sequence, and this variability probably accounts for the observed specificity of the entry exclusion process. For example, the IncI plasmids R144, R64, and ColIb are said to belong to a single exclusion group as they all exclude each other and they encode nearly identical ExcA exclusion proteins (Hartskeerl et al., 1985b). Conversely, the two IncF plasmids R-100 and F do not exclude each other (Willetts and Maule, 1986) and they encode highly diverse TraS entry exclusion proteins. The related ICEs R391 and SXT also do not exclude each other, and distinct amino acid sequences in the C termini of EexS and EexR account for this specificity.

The mechanism(s) of action of entry exclusion proteins are largely unknown. Haase et al. (1996) suggested that the target of TrbK was a component of the donor cell mating pair formation system. Frost and colleagues showed that a *traG* mutant F plasmid complemented with an R100 *traG* was no longer excluded from a recipient producing the F exclusion proteins TraS and TraT; they concluded that the donor cell TraG can distinguish between the distinct F and R100 TraS proteins (Anthony et al., 1999). In a screen to determine the *eex* target in donor cells, we identified the mating pair formation protein TraG as the ICE-encoded component that acts in donor cells to mediate exclusion. Furthermore, targeted changes in *traG* and *eex* revealed some of the determinants of EexS/$TraG_S$- and EexR/$TraG_R$-mediated exclusion. Remarkably, we found that alteration of only three amino acids in TraG switched the donor cell exclusion type. Thus, R391 containing a chimeric TraG with three amino acids in $TraG_R$ switched to the residues found in $TraG_S$ was excluded from a recipient producing EexS and not from a recipient producing EexR. The simplest explanation of these findings is that there is a direct interaction between cognate TraG and Eex proteins that dictates exclusion. We propose that similar interactions between entry exclusion proteins in recipient cells and TraG-like mating pair formation proteins in donor cells establishes the mechanistic basis for other entry exclusion systems. Often genes involved in similar functions reside in close proximity to each other in the genome. This seems to be true for exclusion components, as many entry exclusion genes are found in close proximity to *traG*-like genes. *eexS* and *traS* are adjacent to *traG* orthologs in the SXT and F genomes, respectively, and this gene arrangement appears to be conserved for other entry exclusion genes whose interacting partners have not been described. For example, *eex* is upstream of *traD* in pKM101 and *trbK* is upstream of *trbL* in RP4. Genetic linkage of entry exclusion proteins may facilitate per-

petuation of exclusion in hybrid mobile elements that arise via recombination.

A perplexing question arises from our model of exclusion: how can inner membrane proteins found in different cells interact? The outer membrane of the gram-negative cell wall constitutes a significant obstruction to interactions between inner membrane proteins. Two different scenarios can be invoked to surmount this barrier. First, it is possible that all or part of Eex or TraG is released from the cell and acts outside of the close confines of the mating pair in a fashion analogous to the pheromones produced by certain gram-positive bacteria (Clewell, 1993). This possibility seems unlikely, since it does not explain our observation that *traG* and *eex* must be expressed in donor and recipient cells, respectively, for exclusion to occur; i.e., overexpression of *eexS* in a donor does not influence its capacity to transfer SXT. In a second scenario, Eex and TraG interact within the confines of the mating bridge. The architecture of this conduit for conjugal DNA transfer remains mysterious, so our ideas regarding this scenario are speculative. The N-terminal region of TraG is homologous to the inner membrane protein VirB6 from the Ti plasmid (Lawley et al., 2003), a component of the DNA secretion channel that is required for DNA transport to the cell exterior (Jakubowski et al., 2004). It seems likely that TraG is a part of the SXT secretion channel and that a part of this protein could reach the periplasm of the recipient cell, where it could contact Eex. An alternative proposed by Frost and colleagues is that TraG is translocated to the recipient cell during conjugation (Lawley et al., 2003). We do not yet know how TraG-Eex interaction results in exclusion. This interaction could interfere with TraG's DNA transport function or hinder its association with another recipient component. Eex may act analogously to Imm, a phage T4 inner membrane protein involved in phage superinfection exclusion, which is thought to block DNA transfer across the inner membrane by binding to a virion protein (Lu and Henning, 1994).

Regardless of the molecular mechanism(s) of exclusion, the wide distribution of exclusion systems among conjugative plasmids suggests that these systems confer a benefit to their host elements. It has been proposed that exclusion systems prevent the coexistence of plasmids that utilize identical replication systems, thereby avoiding plasmid loss due to incompatibility (van der Hoeven, 1985). However, this idea is not relevant to nonreplicative ICEs, as they are integrated in the host chromosome. An additional potential benefit of exclusion for both plasmids and ICEs is that these systems may favor the coexistence of different elements rather than identical ones, thereby enabling gene reassortment to generate novel elements. For example, SXT and R391 do not exclude each other's transfer, and the coexistence of these ICEs in the same host has been shown to yield novel elements (Burrus and Waldor, 2004a). Interactions between TraG-like mating pair formation proteins in donors and entry exclusion proteins in recipients underlie a self-recognition system that prevents redundant ICE or plasmid transfer, yet allows coexistence of different elements in the same cell. Such mechanisms for self-recognition play a critical role in promoting gene reassortment and are widespread in nature (Clewell, 1993; Dunny et al., 1978; Kahmann and Bolker, 1996).

Experimental Procedures

Bacterial Strains, Plasmids, and Media

The bacterial strains and plasmids used in this study are described in Supplemental Table S1 (available with this article online). Bacterial strains were grown and maintained as described (Beaber et al., 2002b). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; sulfamethoxazole, 160 μg/ml; trimethoprim, 32 μg/ml; tetracycline, 20 μg/ml.

Plasmid and Strain Constructions

The chimeric *eex* genes *eexRS* and *eexSR* were constructed using Splicing Overlap PCR Extension PCR mutagenesis (Horton et al., 1989). The amino- and carboxy-terminal parts of *eexS* and *eexR* were amplified by PCR. The coding sequence of the amino-terminal fragment from *eexS* was fused with the coding sequence of the carboxy-terminal fragment from *eexR* to create *eexSR* chimera. The *eexSR* chimera was created by fusing the coding sequence of the amino-terminal region from *eexR* with the coding sequence of the carboxy-terminal region from *eexS*. The *eexSR* and *eexRS* chimeric DNA fragments were cloned into pBAD-TOPO (Invitrogen) to create the plasmids pEexSR and pEexRS, respectively. The DNA sequences of the inserts in these constructs were all confirmed by DNA sequencing.

Deletions of *eexS*, *eexR*, $traG_S$, and $traG_R$ were introduced into SXT or R391 through one-step chromosomal gene inactivation as described previously (Datsenko and Wanner, 2000). All deletions were confirmed by PCR. Chimeric *traG* genes were constructed using the λ Red recombination system and a *cat-sacB* cassette as previously described (Yu et al., 2000). The chimeric *traG* gene constructs were confirmed by sequencing. Primer sequences are available upon request.

Bacterial Conjugation and Exclusion Assay

Strains were prepared for mating assays by diluting overnight cultures 1:100 and growing cells for 3 hr. Subsequently, 1 ml of donor and recipient cultures were mixed, spun down, and resuspended in a final volume of 100 μl of LB broth. 100 μl of the mating mixtures were spread onto a membrane filter on an LB plate and incubated for 2 hr. When pTraG was used for transfer, donor cells were grown in the presence of 0.02% glucose for 3 hr and then induced with 0.02% arabinose for 15 min; subsequently, cells were mated for 5 hr. Cells were harvested in 2 ml of LB broth and serial dilutions were plated on the appropriate selective media to determine the numbers of donors and exconjugants. Exclusion indices for SXT (EI_S) and R391 (EI_R) transfer were calculated by dividing the frequency of transfer of the ICE into an ICE-free recipient by the frequency of transfer of the ICE into the recipient of interest.

Cell Fractionation and Protein Analysis

Expression from pEexS-His_6, pTraG$_S$-His_6, pSetR-His_6, and pFrdD-His_6 was induced by adding 0.02% arabinose to cell cultures. Inner and outer membranes were selectively extracted with Triton X-100 using a method described by Russel and Kazmierczak (1993). Samples in 4% SDS were boiled at 95°C for 5 min. Equal protein amounts of each cell fraction were resolved on a 10% SDS-PAGE gel and later transferred to a nitrocellulose membrane. His_6-tagged proteins were detected using an anti-His_5 antibody (Qiagen), and OmpA was detected using rabbit anti-OmpA antibody (Prasadarao et al., 1996).

Supplemental Data

Supplemental Data include one table and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/8/6/963/DC1/.

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References

Achtman, M., Kennedy, N., and Skurray, R. (1977). Cell-cell interactions in conjugating *Escherichia coli*: role of TraT protein in surface exclusion. Proc. Natl. Acad. Sci. USA *74*, 5104–5108.

Achtman, M., Manning, P.A., Kusecek, B., Schwuchow, S., and Willetts, N. (1980). A genetic analysis of F sex factor cistrons needed for surface exclusion in *Escherichia coli*. J. Mol. Biol. *138*, 779–795.

Anthony, K.G., Klimke, W.A., Manchak, J., and Frost, L.S. (1999). Comparison of proteins involved in pilus synthesis and mating pair stabilization from the related plasmids F and R100–1: insights into the mechanism of conjugation. J. Bacteriol. *181*, 5149–5159.

Beaber, J.W., and Waldor, M.K. (2004). Identification of operators and promoters that control SXT conjugative transfer. J. Bacteriol. *186*, 5945–5949.

Beaber, J.W., Burrus, V., Hochhut, B., and Waldor, M.K. (2002a). Comparison of SXT and R391, two conjugative integrating elements: definition of a genetic backbone for the mobilization of resistance determinants. Cell. Mol. Life Sci. *59*, 2065–2070.

Beaber, J.W., Hochhut, B., and Waldor, M.K. (2002b). Genomic and functional analyses of SXT, an integrating antibiotic resistance gene transfer element derived from *Vibrio cholerae*. J. Bacteriol. *184*, 4259–4269.

Burrus, V., and Waldor, M.K. (2004a). Formation of SXT tandem arrays and SXT-R391 hybrids. J. Bacteriol. *186*, 2636–2645.

Burrus, V., and Waldor, M.K. (2004b). Shaping bacterial genomes with integrative and conjugative elements. Res. Microbiol. *155*, 376–386.

Clewell, D.B. (1993). Bacterial Conjugation (New York: Plenum Press).

Coetzee, J.N., Datta, N., and Hedges, R.W. (1972). R factors from *Proteus rettgeri*. J. Gen. Microbiol. *72*, 543–552.

Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA *97*, 6640–6645.

Dunny, G.M., Brown, B.L., and Clewell, D.B. (1978). Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci. USA *75*, 3479–3483.

Finlay, B.B., and Paranchych, W. (1986). Nucleotide sequence of the surface exclusion genes *traS* and *traT* from the IncF0 *lac* plasmid pED208. J. Bacteriol. *166*, 713–721.

Firth, N., and Skurray, R. (1992). Characterization of the F plasmid bifunctional conjugation gene, *traG*. Mol. Gen. Genet. *232*, 145–153.

Freudl, R., Schwarz, H., Klose, M., Movva, N.R., and Henning, U. (1985). The nature of information, required for export and sorting, present within the outer membrane protein OmpA of *Eschericia coli* K-12. Mol. Gen. Genet. *201*, 76–81.

Furuya, N., and Komano, T. (1994). Surface exclusion gene of IncI1 plasmid R64: nucleotide sequence and analysis of deletion mutants. Plasmid *32*, 80–84.

Haase, J., Kalkum, M., and Lanka, E. (1996). TrbK, a small cytoplasmic membrane lipoprotein, functions in entry exclusion of the IncP alpha plasmid RP4. J. Bacteriol. *178*, 6720–6729.

Hartskeerl, R.A., and Hoekstra, W.P. (1984). Exclusion in IncI-type *Escherichia coli* conjugations: the stage of conjugation at which exclusion operates. Antonie Van Leeuwenhoek *50*, 113–124.

Hartskeerl, R., Tommassen, J., and Hoekstra, W. (1985a). Relationship between the proteins encoded by the exclusion determining locus of the IncI plasmid R144 and the cellular localization of these proteins in *Escherichia coli* K-12. Mol. Gen. Genet. *200*, 138–144.

Hartskeerl, R., Zuidweg, E., van Geffen, M., and Hoekstra, W. (1985b). The IncI plasmids R144, R64 and ColIb belong to one exclusion group. J. Gen. Microbiol. *131*, 1305–1311.

Hochhut, B., and Waldor, M.K. (1999). Site-specific integration of the conjugal *Vibrio cholerae* SXT element into *prfC*. Mol. Microbiol. *32*, 99–110.

Hochhut, B., Beaber, J.W., Woodgate, R., and Waldor, M.K. (2001). Formation of chromosomal tandem arrays of the SXT element and R391, two conjugative chromosomally integrating elements that share an attachment site. J. Bacteriol. *183*, 1124–1132.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., and Pease, L.R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene *77*, 61–68.

Jakubowski, S.J., Krishnamoorthy, V., Cascales, E., and Christie, P.J. (2004). *Agrobacterium tumefaciens* VirB6 domains direct the ordered export of a DNA substrate through a type IV secretion system. J. Mol. Biol. *341*, 961–977.

Jalajakumari, M.B., Guidolin, A., Buhk, H.J., Manning, P.A., Ham, L.M., Hodgson, A.L., Cheah, K.C., and Skurray, R.A. (1987). Surface exclusion genes *traS* and *traT* of the F sex factor of *Escherichia coli* K-12. Determination of the nucleotide sequence and promoter and terminator activities. J. Mol. Biol. *198*, 1–11.

Kahmann, R., and Bolker, M. (1996). Self/nonself recognition in fungi: old mysteries and simple solutions. Cell *85*, 145–148.

Lawley, T.D., Klimke, W.A., Gubbins, M.J., and Frost, L.S. (2003). F factor conjugation is a true type IV secretion system. FEMS Microbiol. Lett. *224*, 1–15.

Lu, M.J., and Henning, U. (1994). Superinfection exclusion by T-even-type coliphages. Trends Microbiol. *2*, 137–139.

Manning, P.A., Morelli, G., and Achtman, M. (1981). TraG protein of the F sex factor of *Escherichia coli* K-12 and its role in conjugation. Proc. Natl. Acad. Sci. USA *78*, 7487–7491.

Nakai, K., and Kanehisa, M. (1991). Expert system for predicting protein localization sites in gram-negative bacteria. Proteins *11*, 95–110.

Ou, J.T. (1975). Mating signal and DNA penetration deficiency in conjugation between male *Escherichia coli* and minicells. Proc. Natl. Acad. Sci. USA *72*, 3721–3725.

Pohlman, R.F., Genetti, H.D., and Winans, S.C. (1994). Entry exclusion of the IncN plasmid pKM101 is mediated by a single hydrophilic protein containing a lipid attachment motif. Plasmid *31*, 158–165.

Prasadarao, N.V., Wass, C.A., Weiser, J.N., Stins, M.F., Huang, S.H., and Kim, K.S. (1996). Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. Infect. Immun. *64*, 146–153.

Russel, M., and Kazmierczak, B. (1993). Analysis of the structure and subcellular location of filamentous phage pIV. J. Bacteriol. *175*, 3998–4007.

Samuels, A.L., Lanka, E., and Davies, J.E. (2000). Conjugative junctions in RP4-mediated mating of *Escherichia coli*. J. Bacteriol. *182*, 2709–2715.

Singer, M., Baker, T.A., Schnitzler, G., Deischel, S.M., Goel, M., Dove, W., Jaacks, K.J., Grossman, A.D., Erickson, J.W., and Gross, C.A. (1989). A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbiol. Rev. *53*, 1–24.

Tusnady, G.E., and Simon, I. (2001). The HMMTOP transmembrane topology prediction server. Bioinformatics *17*, 849–850.

van der Hoeven, N. (1985). Evolution of bacterial surface exclusion against incompatible plasmids. J. Theor. Biol. *117*, 431–452.

Waldor, M.K., Tschape, H., and Mekalanos, J.J. (1996). A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. J. Bacteriol. *178*, 4157–4165.

Westenberg, D.J., Gunsalus, R.P., Ackrell, B.A., Sices, H., and Cecchini, G. (1993). *Escherichia coli* fumarate reductase *frdC* and *frdD* mutants. Identification of amino acid residues involved in catalytic activity with quinines. J. Biol. Chem. *268*, 815–822.

Willetts, N., and Maule, J. (1986). Specificities of IncF plasmid conjugation genes. Genet. Res. *47*, 1–11.

Yu, D., Ellis, H.M., Lee, E.C., Jenkins, N.A., Copeland, N.G., and Court, D.L. (2000). An efficient recombination system for chromosome engineering in *Escherichia coli*. Proc. Natl. Acad. Sci. USA *97*, 5978–5983.